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INSTRUMENTS AND TECHNIQUES

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# A rapid enzymatic method for the isolation of defined kidney tubule fragments from mouse

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**Keywords** Mouse kidney · Isolated renal tubules · pH measurement · Vacuolar $H^+$-ATPase · Collecting duct

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## Introduction

The increasing use of genetically engineered mice has led to the reintroduction of old techniques as well as the need to develop new tools to investigate altered organ and cell function in these animals [28, 32, 39]. Complex organs such as the kidney, with a variety of functionally and morphologically different compartments and cell types, impose additional difficulties. Numerous techniques, such as whole isolated kidney perfusion [25], in situ microperfusion of single nephron segments in the intact kidney [39], biochemical and transport studies in tubule suspensions [15, 45, 49] or isolated membrane vesicles [29, 35], isolation of kidney tubules [14, 15, 31, 36, 38], kidney slices [2, 3, 5] and preparations of cell cultures [4, 21, 23, 41, 44] have been developed and used to investigate kidney organ function as well as segment- or cell-specific functions. The preparation of isolated kidney tubule fragments allowed for the first time access to all nephron segments and the controlled examination of transport

processes. Non-perfused isolated tubule fragments have proven to be an excellent preparation for studying hormone action on intracellular second messengers or for performing electrophysiological measurements (patch-clamp or intracellular or -luminal electrodes) [7, 8, 22, 36, 38]. In combination with perfusion of the isolated tubule, polarized transport can be analyzed [19, 38]. Several methods have been employed to obtain isolated tubule fragments from salamander, dog, rabbit, rat, mouse, and man by hand dissection, enzymatic treatment or a combination of both [13, 14, 17, 18, 19, 31, 36, 38]. Many of the initial studies using isolated perfused tubules were performed on rabbit tubules using manual dissection techniques. Proximal, thin and thick limb as well as distal convoluted and cortical collecting duct were all isolated and perfused in vitro. Although the mouse has been used successfully in the past, these studies have been confined to only relatively few segments. Schafer et al. have described a new simple method for digesting rat kidney slices to prepare large amounts of defined nephron segments [38]. We have also employed this method, with slight modifications, for studying the regulation of $H^+$-ATPase function in rat proximal tubule fragments [51]. To be able to study transport processes in various nephron segments from normal and genetically altered mice, we have now adapted and modified this enzymatic method to isolate large quantities of fragments from defined nephron segments from the mouse kidney rapidly and highly reproducibly. The mouse tubules isolated using this technique were examined morphologically by light and electron microscopy, and by immunofluorescence using antibodies specific for several acid-base transporting proteins. In addition, proximal tubules, cortical collecting ducts, and inner medullary collecting ducts were characterized functionally using the intracellular pH ($pH_i$) indicator 2′,7′-bis(2carboxylethyl)-5(6)-carboxyfluorescein (BCECF).

The proximal tubule and the cortical collecting duct play a key role in the transport of protons and bicarbonate and in the regulation of acid-base homeostasis by the kidney [20]. A variety of transport proteins in these segments are involved in proton secretion ($Na^+/H^+$ exchangers, $H^+$-ATPases, and $H^+/K^+$-ATPase) or bicarbonate transport ($Na^+/HCO_3^-$ transporters and $Cl^-/HCO_3^-$ exchanger [11, 17, 19, 20, 51, 55]. The bulk of proton secretion occurs through apical $Na^+/H^+$ exchangers and an $H^+$-ATPase in the proximal tubule [9, 19, 20, 55], whereas proton secretion in the intercalated cells of the cortical collecting duct, and later in the outer medullary collecting duct, depends mainly on an $H^+$-ATPase [9, 20, 40, 50]. The role of an $H^+/K^+$-ATPase has not been clarified fully [20, 50]. In the present study, we measured total $Na^+$-independent and -dependent $pH_i$ recovery rates in the proximal tubule (S1/S2 segments) and in intercalated cells in the cortical collecting duct and outer medullary collecting duct, as markers for the functional integrity of the selected tubule fragments obtained.

## Materials and methods

### Enzymatic preparation of tubules

Male C57BL/6 J mice (Jackson Laboratory, Maine, USA, 25–35 g body weight) were sacrificed with pentobarbital (150 mg/kg), both kidneys rapidly removed and transferred into ice-cold HEPES solution (see Table 1). After removal of the capsula, vessels, and pelvis, the kidneys were cut into coronal slices 2–3 mm in thickness. Usually three or four slices from the middle of each kidney could be obtained due to the thickness of the cortex and medulla. For the preparation of cortical or medullary tubules, 2- to 3-mm slices of the respective region were prepared under a stereo microscope and transferred into the pre-warmed digestion solution. Cortical or medullary tissue was then incubated in a digestion solution containing 4 ml MEM (GibcoBRL), 5 mM glycine, 6 mg/ml trypsin inhibitor type II-S (Sigma T-9128), and 250 μg/ml collagenase (Sigma C-9891), pH 7.4 at 37 °C in a water bath for approximately 15 min without shaking. The digestion period depended on the age and size of the animal, increased times were used for larger and older mice. When the medium became cloudy upon gentle shaking, the digestion was stopped by transferring the tubules to ice, carefully removing the supernatant and replacing it with 4 ml ice-cold 1% BSA-HEPES solution. The BSA HEPES solution was replaced with ice-cold HEPES solution after 10 min and tubules were maintained on ice until use.

### Selection of tubules for experiments

Isolated tubules were selected at 4 °C under a stereo microscope after 0.5 ml of the tubule suspension had been diluted in 10 ml of ice-cold HEPES solution. Tubules were selected using the following criteria: the nephron segment had (1) to be identifiable; (2) to consist of only one identifiable segment; (3) to be in one continuous piece without any visible damage; (4) to have a minimum length of

**Table 1** Composition of solutions (in mM) used for functional experiments. All solutions were titrated to pH 7.4 at 37 °C using either NaOH or KOH. *N*-Methyl-D-glucamine (*NMDG*) was titrated with HCl

| | Standard HEPES | $Na^+$-free HEPES | $Na^+$-free HEPES+$NH_4Cl$ | $Na^+$ and $K^+$-free HEPES | High $K^+$ calibration |
|---|---|---|---|---|---|
| NaCl | 125 | – | – | – | – |
| NMDG | – | 125 | 105 | 130 | 32.8 |
| $NH_4Cl$ | – | – | 20 | – | – |
| KCl | 3 | 3 | 3 | 3 | 105 |
| MgSO4 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 |
| CaCl2 | 1 | 1 | 1 | 1 | 1 |
| $KH_2PO_4$ | 2 | 2 | 2 | 2 | – |
| Glucose | 5 | 5 | 5 | 5 | – |
| HEPES | 32.2 | 32.2 | 32.2 | 32.2 | 32.2 |
| pH | 7.4 | 7.4 | 7.4 | 7.4 | 6.0, 7.0, 8.0 |

300 μm; (5) to have no other visible connective tissue attached. Viability of the isolated tubules was determined by their ability to exclude trypan blue and by the rate of $pH_i$ recovery following an acute acid load (see below). Proximal tubules were viable for 1–2 h after stopping the digestion procedure whereas the collecting duct segments remained viable for 5–6 h. Thus, initial experiments following digestion were typically conducted on the proximal tubules followed by studies on the collecting duct.

### Plasma membrane intactness of tubules

The intactness of the plasma membrane of the optically selected tubules was assessed by their ability to exclude trypan blue. Tubules were transferred onto cover-slips coated with biological adhesive (Cell-Tak, Becton-Dickinson). A trypan blue solution (0.04% diluted with the normal Ringer solution) was then added for 10 min, followed by extensive washing with normal Ringer. Damaged cells, identified as those that remained stained, were counted. From each preparation of tubules, damaged cells were counted in ten random fields using a 40× objective. One field contained 20–30 tubule fragments, seven separate preparations from different animals were stained and counted.

### Immunocytochemistry

Isolated tubules were transferred onto cover-slips prepared with polylysine and allowed to adhere for 15 min. After fixation with paraformaldehyde-lysine-periodate solution (PLP) [27] overnight at 4 °C, tubules were washed 3 times with PBS, permeabilized with 0.1% Triton X, washed twice with PBS, treated with 1% SDS [12], washed 3 times with PBS, and incubated with PBS containing 1% bovine serum albumin for 15 min prior to addition of the primary antibodies. The primary antibodies were diluted in PBS and applied overnight at 4 °C: goat anti-human aquaporin-2 (AQP-2, 1:200 purified antibody, Santa Cruz, [52]), rabbit anti-mouse pendrin (1:500 raw serum, [52]), rabbit anti-rat anion exchanger-1 (AE-1, 1:200 affinity purified, Chemicon), rabbit anti-bovine ATP6V1B1 (B1) (1:200 raw serum, [30] kindly provided by K. E. Finberg, Yale University), rabbit anti-human ATP6V0A4 (a4) (1:1000 raw serum). The anti-a4 serum was produced in rabbit using a C-terminal peptide of the human sequence which had a N-terminally introduced cysteine for linkage to keyhole limpet hemocyanin (KLH, $NH_2$-CKFSPFSFKHILDGTAEE-COOH, Pineda antibody service, Berlin, Germany). The anti-a4 serum showed the same pattern of staining in whole mouse kidney as described previously [42]. No staining was observed for any antibody or serum using only pre-immune sera or preabsorbing the sera with the immunizing peptides, respectively (data not shown). Tubules were then washed twice for 5 min with high-NaCl PBS (PBS+2.7% NaCl), once with PBS, and incubated with the secondary antibodies (donkey anti-goat Alexa 488, donkey anti-rabbit Alexa 594, Molecular Probes, Eugene, Ore., USA) at a dilution of 1:400 and 1:1,000, respectively, for 1 h at room temperature. Tubules were again washed twice with high-NaCl PBS and once with PBS. Cover-slips were mounted with Vectashield mounting medium diluted 1:1 in TRIS-HCl pH 8.9, slides were examined using a Leica SP1 confocal microscope, and the images assembled with Photoshop (Adobe, San Jose, Calif., USA) software.

### Electron microscopy

Enzymatically digested tubules were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate [Electron Microscopy Sciences (EMS), Ft. Washington, Pa., USA], pH 7.4. They were then rinsed in cacodylate buffer, 3 times for 5 min each time. The tubules were post-fixed in 1% osmium tetroxide (EMS) in 0.1 M cacodylate buffer for 1 h at room temperature, and then rinsed as above. The samples were dehydrated through a graded ethanol series to 100 % ethanol, then rinsed in propylene oxide and infiltrated with Epon (EMS) in a solution of Epon:propylene oxide (1:1), overnight. The next day, they were placed in fresh Epon for several hours and then embedded in Epon overnight at 60 °C. Ultrathin sections were cut on a Reichert Ultracut E ultramicrotome. They were collected on Formvar-coated slot grids, stained with lead citrate and uranyl acetate, and examined in a Philips CM 10 transmission electron microscope at 80 kV.

### pHi measurements

For $pH_i$ measurements, selected tubules were transferred to a perfusion chamber containing cover-slips precoated with the cell adhesive Cell-Tak (Becton-Dickinson). The temperature of the chamber was maintained at 37±0.5 °C by an electronic feedback circuit. The control bath solution was initially a standard HEPES solution (Table 1), flowing continuously at ≈3 ml/min. The chamber volume was ≈180 μl. Single tubule fragments were loaded with the acetoxymethyl ester of the pH-sensitive dye BCECF (10 μM) for 15 min as described elsewhere [19, 51]. $pH_i$ was measured microfluorometrically by exciting the dye with a 10-μm-diameter spot of light alternately at 490 and 440 nm while monitoring the emission at 532 nm with a video-imaging system [19, 51].

The solutions used are given in Table 1. To measure $H^+$-ATPase activity, bicarbonate-free solutions were used and $Na^+$ removed to abolish $Na^+/H^+$ exchanger activity. For these experiments $Na^+$ was replaced by *N*-methyl-D-glucamine (NMDG). To induce a strong intracellular acidification and activate $H^+$-ATPase, the $NH_4Cl$ prepulse technique was used in the absence of $Na^+$ as described previously [33]. Intercalated cells in the cortical collecting duct and in inner stripe of the outer medullary collecting duct were identified on the basis of their morphology (bulging into the lumen, darker appearance due to their higher content of mitochondria [40]) and their rapid loading with BCECF. All chemicals were obtained from Sigma.

Each experiment was calibrated for $pH_i$ using the nigericin/high-$K^+$ method [47] and the obtained ratios were converted to $pH_i$. $Na^+$-independent $pH_i$ recovery rates in response to an acid load were calculated in proximal tubules in the pH range 6.70–6.80, in the cortical collecting duct in the pH range 6.50–6.60, and in the inner medullary collecting duct from pH 6.50–6.60. $Na^+$-dependent $pH_i$ recovery was measured in proximal tubules in the pH range 6.80–7.00, in the cortical collecting duct in the pH range 6.50–7.00, and in the inner medullary collecting duct from pH 6.70–7.00. The pH ranges chosen represented the average values found in the respective tubule segment.

Data are provided as means±SEM, *n* represents the number of tubules and cells, investigated. The significance of differences between means was established using Students *t*-test, with $P<0.05$ being considered significant.

## Results

### Morphology

Using the modified collagenase digestion technique, single isolated tubule fragments from the outer cortex or inner stripe of the outer medulla were obtained (Fig. 1). The trypan exclusion test was used to evaluate plasma membrane intactness in isolated identifiable tubule fragments which were selected by length, and their lack of attachment to other nephrons or cellular material. Most of the trypan blue-stained tubules were proximal tubules, indicating that they were especially susceptible to membrane damage during the isolation procedure (between 60–75% of the unselected proximal tubules had more than

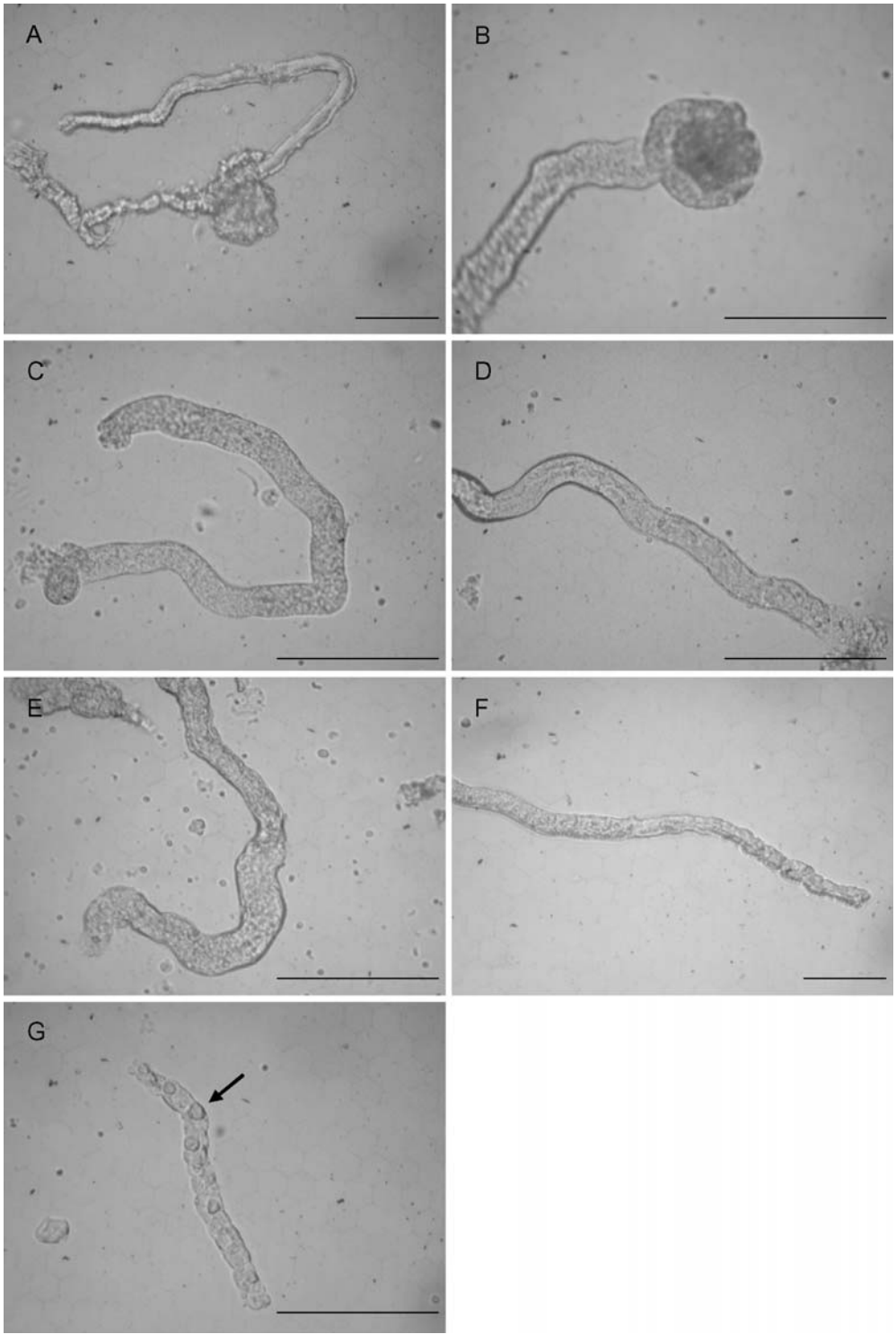


**Fig. 1A–G** Phase contrast micrographs of different fragments of mouse tubules obtained by digestion (*bar* 20 μm for all micrographs). **A** Glomerulus with attached vessels, **B** glomerulus and proximal tubule S1 segment, **C** proximal tubule fragment, **D** S3 proximal tubule fragment, **E** S3 proximal tubule and thin descending limb, **F** distal tubule and thick ascending limb from the inner medulla, **G** cortical collecting duct with prominent intercalated cells (*arrow*)

three cells stained with trypan blue, $n$=7 preparations). Only a few distal tubule and cortical collecting duct fragments were stained, suggesting that they were more robust (10–20%, $n$=7 preparations). Electron microscopy was used to examine the morphology of both selected and unselected tubule fragments further. As shown in Fig. 2A, a large number of unselected proximal tubules were damaged and exhibited condensed mitochondria, vacuoles, and loss of cellular integrity. However, some proximal tubules showed little or no damage (Fig. 2B). Figure 2 also shows examples of undamaged fragments of the distal tubule and cortical collecting duct.

Selected isolated cortical and medullary collecting duct fragments were immunostained to localize several transport proteins involved in acid-base transport in these nephron segments (Fig. 3A–D). Two subunits of the vacuolar $H^+$-

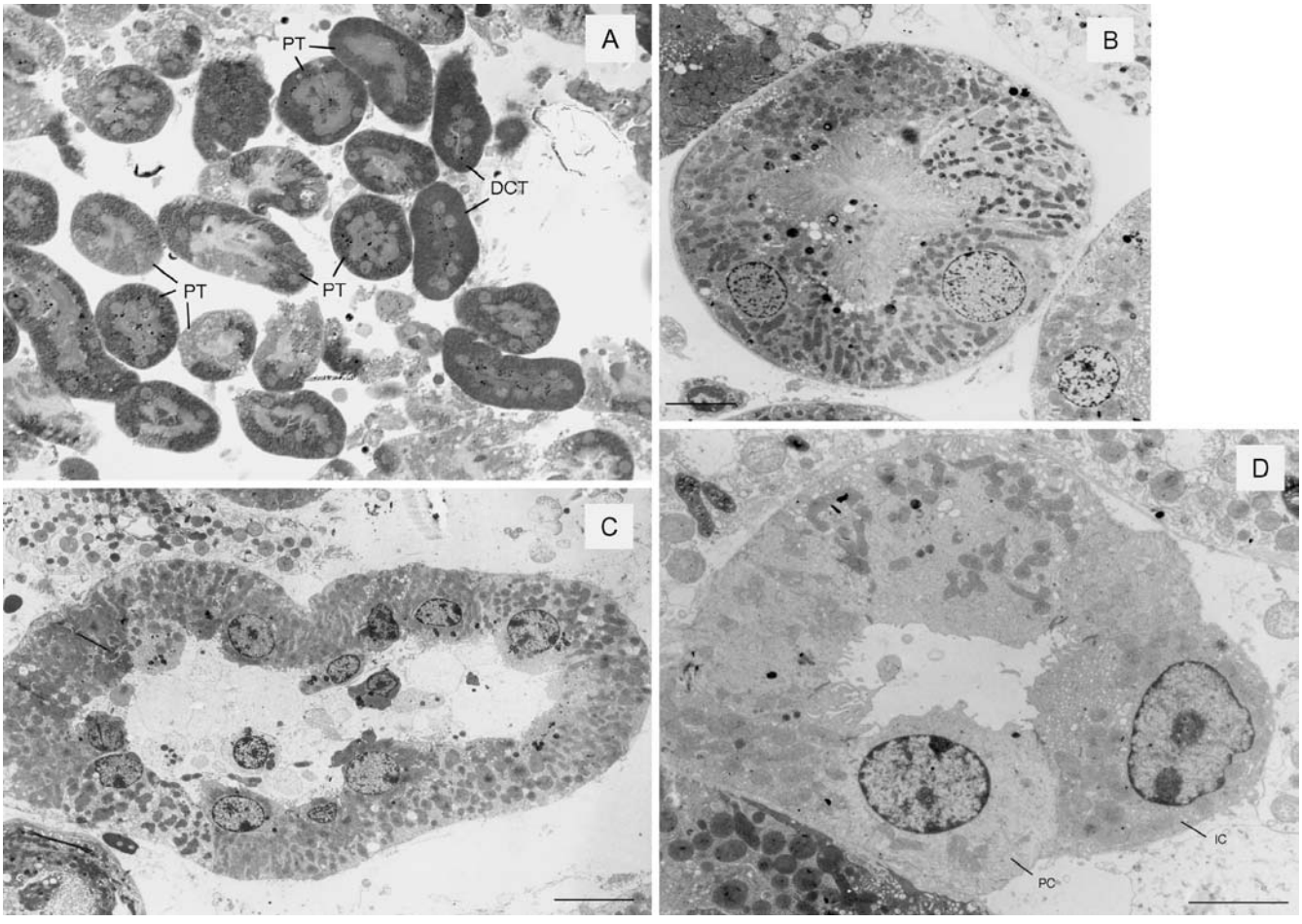


**Fig. 2A–D** Electron micrographs of mouse tubules obtained by digestion of cortical slices. **A** Overview showing unselected fragments of proximal (*PT*) and distal convoluted tubules (*DCT*). Many of the proximal tubules are damaged (*bar* 20 μm). **B** Undamaged proximal tubule (*bar* 5 μm), **C** distal tubule (*bar* 5 μm), **D** cortical collecting duct with intercalated cell (*IC*) and principal cell (*PC*) (*bar* 5 μm)

ATPase, ATP6V0A4 (a4) and ATP6V1B1 (B1), the $Cl^-/HCO_3^-$ exchanger band 3/AE-1, the $Cl^-$/anion exchanger pendrin, and the principal cell-specific water channel AQP-2 were stained. ATP6V0A4 and ATP6V1B1 staining was found both in the membrane as well as in cytoplasmic vesicles, as described earlier in the intact rat or mouse kidney for other subunits of the proton pump [11, 24, 46]. In addition, the type-A intercalated cell-specific AE-1 exchanger and the non-type A intercalated cell-specific $Cl^-$/anion exchanger pendrin showed a clear basolateral and apical distribution, respectively, as described in several species, indicating that the polarity of expression had been maintained in our preparation [1, 48, 52, 54].

## $pH_i$ measurements

Measurements of $pH_i$ were used to demonstrate the functional integrity of selected tubule fragments obtained by digestion. In the absence of $HCO_3^-$ the initial $pH_i$ in the digested proximal tubule S1/S2 fragments was 7.28±0.01 (Table 2). $pH_i$ decreased after the complete removal of extracellular $Na^+$ from the bath by 0.31±0.01 pH units to 6.96±0.02. Further acidification was induced by the $NH_4Cl$ (20 mM) pulse technique as described previously [33]. After removal of $NH_4Cl$ and in the continued absence of extracellular $Na^+$ a slow alkalinization was observed (0.021±0.002 pH units/min, $n$=55, Fig. 4A, C), which could be blocked completely by the specific vacuolar $H^+$-ATPase inhibitor concanamycin (100 nM) [16] (0.001±0.001 pH units/min, $n$=32, Fig. 4B, C, Table 2). Addition of extracellular $Na^+$ caused rapid alkalinization (0.144±0.008 pH units/min, $n$=55), which was not affected by concanamycin (0.141±0.018, $n$=32, Fig. 4D).

Single intercalated cells in digested cortical collecting duct fragments, had a resting $pH_i$ of 7.26±0.01 ($n$=53) in the absence of $HCO_3^-$. Removal of extracellular $Na^+$ led to intracellular acidification to pH 7.04±0.01. $pH_i$ fell further to 6.27±0.02 after an $NH_4Cl$ pulse (20 mM). In the continued absence of extracellular $Na^+$ $pH_i$ recovered slowly (0.037±0.002 pH units/min, $n$=53, Fig. 5A, C, Table 2) following $NH_4Cl$ removal. In the presence of $Na^+$ $pH_i$ recovered at 0.182±0.013 pH units/min ($n$=53,

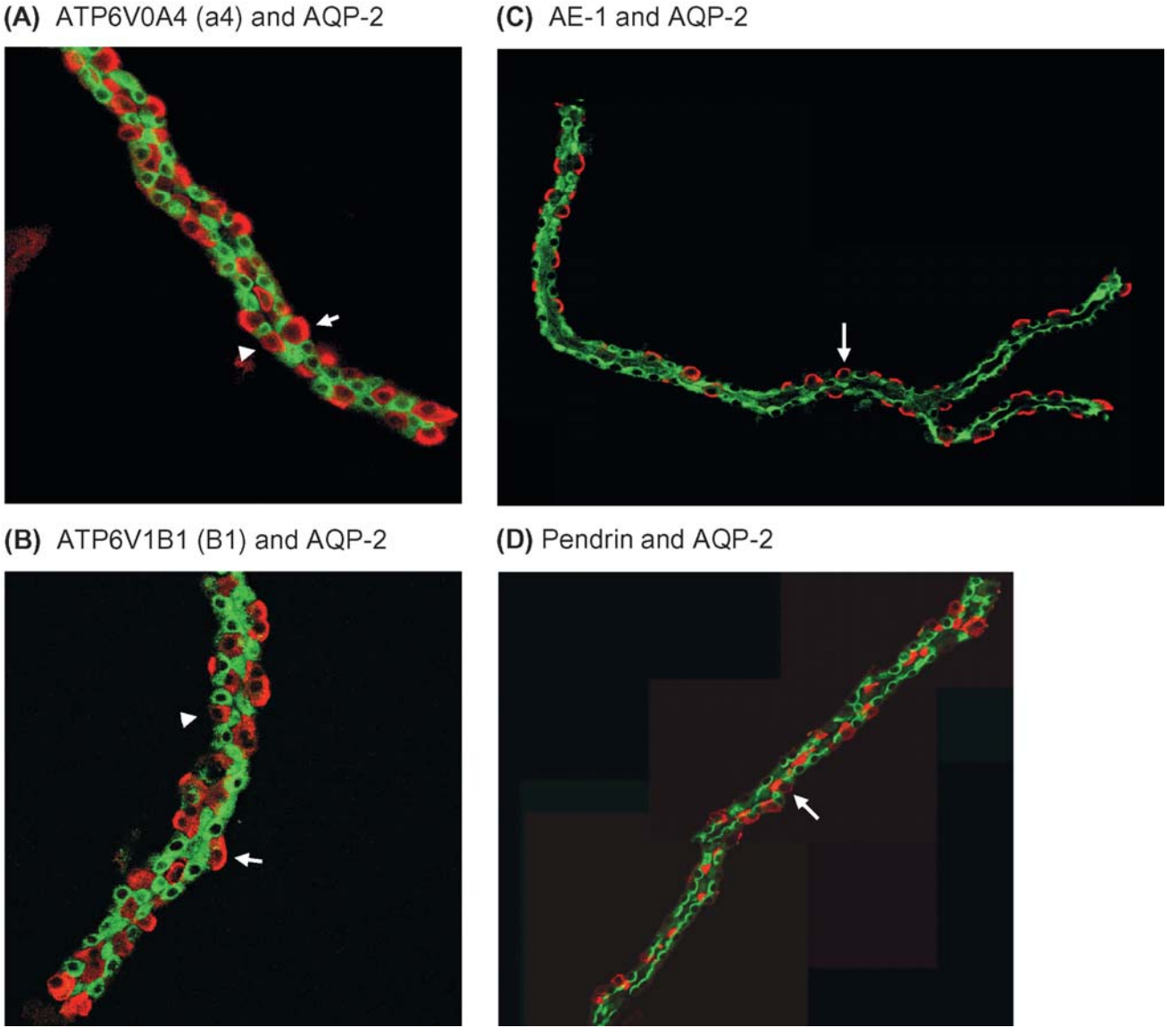


**Fig. 3A–D** Localization of acid-base transporting proteins in cortical and medullary collecting ducts obtained by digestion. Digested mouse cortical and medullary collecting ducts were labeled with antibodies against the principal cell-specific water channel aquaporin-2 (AQP-2, *green*), against two subunits of the vacuolar $H^+$-ATPase *ATP6V0A4 (a4)* and *ATP6V1B1 (B1)*, and against anion exchanger-1 (*AE-1*) or pendrin, and viewed with confocal microscopy. **A**, **B** Expression of both vacuolar $H^+$-ATPase subunits in cortical collecting duct fragments was restricted to cells negative for AQP-2 as described previously [30, 42] thus representing intercalated cells. In many cells expression of the vacuolar $H^+$-ATPase subunits was basolateral (*arrow*) or diffuse. Some cells also showed apical staining (*arrow head*). **C** Localization of the band 3/AE-1 $Cl^-/HCO_3^-$ exchanger in cortical and medullary collecting duct. The expression of AE-1 is restricted to the basolateral side of intercalated cells as described in intact kidney marking these cells as type-A intercalated cells. **D** Apical localization of the $Cl^-$/anion exchanger pendrin in intercalated cells. In the intact kidney this protein is expressed apically in non-type A intercalated cells [34, 52, 54]. The basolateral and apical localization of AE-1 and pendrin in intercalated cells, respectively, suggests that polarity of expression of transport proteins is not altered in isolated tubule fragments obtained by rapid digestion. Original magnification: 400×

Fig. 5A, D, Table 2). About 85% of the $Na^+$-independent $pH_i$ recovery was inhibited by the vacuolar $H^+$-ATPase blocker concanamycin (100 nM) (0.005±0.001 pH units/min, Fig. 5B, C, Table 2) suggesting that recovery was due mainly to $H^+$-ATPase activity. Interestingly, the $Na^+$-dependent $pH_i$ recovery rate increased significantly in the presence of concanamycin (0.287±0.017 pH units/min, Fig. 5D, Table 2).

Finally, we examined collecting ducts obtained by digestion from the inner stripe of the outer medulla. Similar to the previous segments, removal of extracellular $Na^+$ acidified single intercalated cells from pH 7.25±0.01 to 7.04±0.02. The slow alkalinization after $NH_4Cl$-induced acidification was, however, only partly blocked by the $H^+$-ATPase inhibitor concanamycin (100 nM) suggesting an additional $H^+$-extruding mechanism such as

**Table 2** Summary of intracellular pH ($pH_i$) measurements in proximal tubules, cortical collecting ducts, and inner medullary collecting ducts

| | Initial $pH_i$ | $Na^+$-independent $pH_i$ recovery ($\Delta$pH/min) | $Na^+$-dependent $pH_i$ recovery ($\Delta$pH/min) | Final $pH_i$ | Number of cells (tubules) |
|---|---|---|---|---|---|
| Proximal tubule S1/S2; control | 7.28±0.01 | 0.021±0.002 | 0.144±0.008 | 7.18±0.02 | 55 (6) |
| Proximal tubule S1/S2 concanamycin | 7.29±0.01 | 0.001±0.001 | 0.141±0.018 | 7.16±0.02 | 32 (4) |
| Cortical collecting duct; control | 7.26±0.01 | 0.037±0.002 | 0.182±0.013 | 7.22±0.01 | 53 (6) |
| Cortical collecting duct; concanamycin | 7.29±0.02 | 0.005±0.001 | 0.287±0.017 | 7.24±0.02 | 40 (4) |
| Outer medullary collecting duct (inner stripe); control | 7.25±0.01 | 0.033±0.002 | 0.112±0.010 | 7.12±0.04 | 36 (4) |
| Outer medullary collecting duct (inner stripe); concanamycin | 7.27±0.01 | 0.017±0.002 | 0.159±0.012 | 7.24±0.03 | 25 (4) |

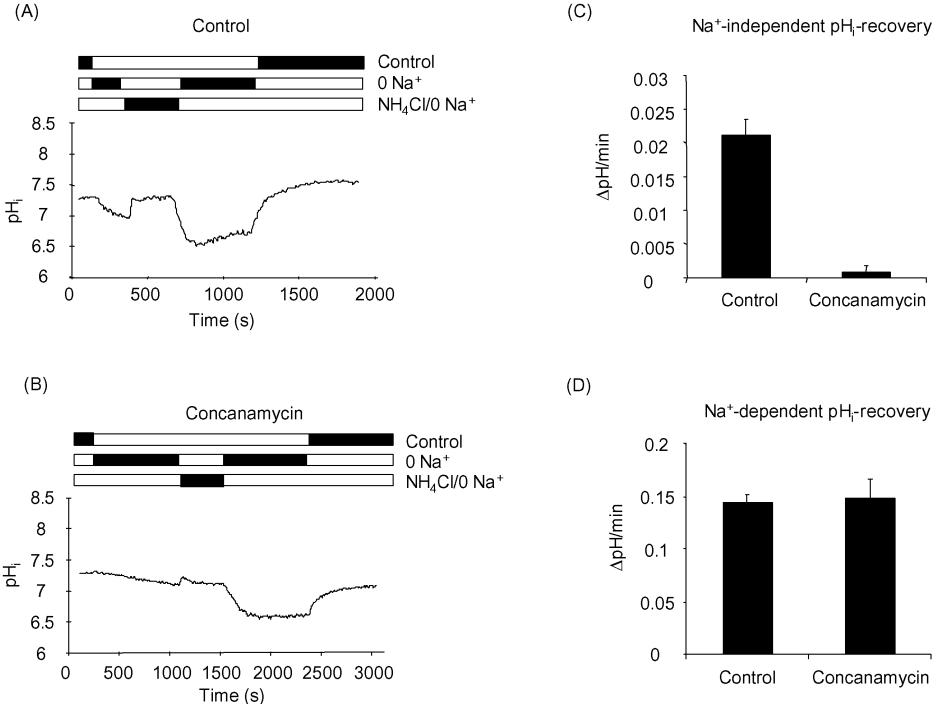


**Fig. 4A–D** $Na^+$-independent and -dependent intracellular pH ($pH_i$) recovery in the proximal tubule S1/S2 segment. **A** Original $pH_i$ tracing from a S1/S2 segment obtained by digestion under control conditions. **B** Original $pH_i$ tracing from a S1/S2 segment in the presence of the specific vacuolar type $H^+$-ATPase inhibitor concanamycin (100 nM). **C** $H^+$-ATPase activity in S1/S2 segments: inhibition of $Na^+$-independent $pH_i$ recovery by the specific vacuolar $H^+$-ATPase blocker concanamycin (100 nM). **D** Summary of $Na^+$-dependent $pH_i$ recovery rates in S1/S2 segments under control conditions and in the presence of concanamycin

an $H^+/K^+$-ATPase. $pH_i$ alkalinization in the absence of concanamycin was 0.033±0.002 pH units/min ($n$=36) and 0.017±0.002 pH units/min in the presence of 100 nM concanamycin ($n$=24). Again, the $Na^+$-dependent $pH_i$ recovery rate increased significantly in the presence of concanamycin (0.112±0.010 vs. 0.159±0.012 pH units/min, Fig. 6D, Table 2).

## Discussion

Over the past few decades, considerable efforts have been made to develop methods for studying tubular and cellular function in isolated kidney tubules or cells (e.g. [7, 14, 15, 18, 22, 28, 36, 38, 45, 49]). Currently, several techniques are employed for digesting kidneys to obtain single cells of defined segmental origin for cell culture, e.g. flow-cytometric immunodissection or gradient centrifugation [4, 21, 23, 41, 44]. However, it is also well documented that cells taken into primary culture may loose their polarity and can potentially up- and down-regulate receptors, transporters, and other membrane proteins, limiting the relevance of these preparations for the study of cell physiology. Similarly, in vivo microperfusion of tubules in the intact kidney has limitations as it does not allow access to all segments of the nephron, thus, making it applicable only to restricted, site-specific questions [28, 39]. The study of freshly isolated kidney tubules in vitro is therefore a complementary technique allowing access to virtually all segments of the nephron with the possibility of controlling conditions on both the luminal and basolateral sides of the perfused tubule [14, 37, 38]. Several techniques using digestion, hand dissection, or a combination of both, are employed currently to obtain isolated tubule segments. Schafer et al. have described a method for enzymatic digestion of the rat kidney isolating large amounts of defined nephron segments that may be used for Western, RT-PCR or experiments not requiring perfusion [38]. We have also employed this method for studying the regulation of $H^+$-ATPase function in rat proximal tubule fragments [51].

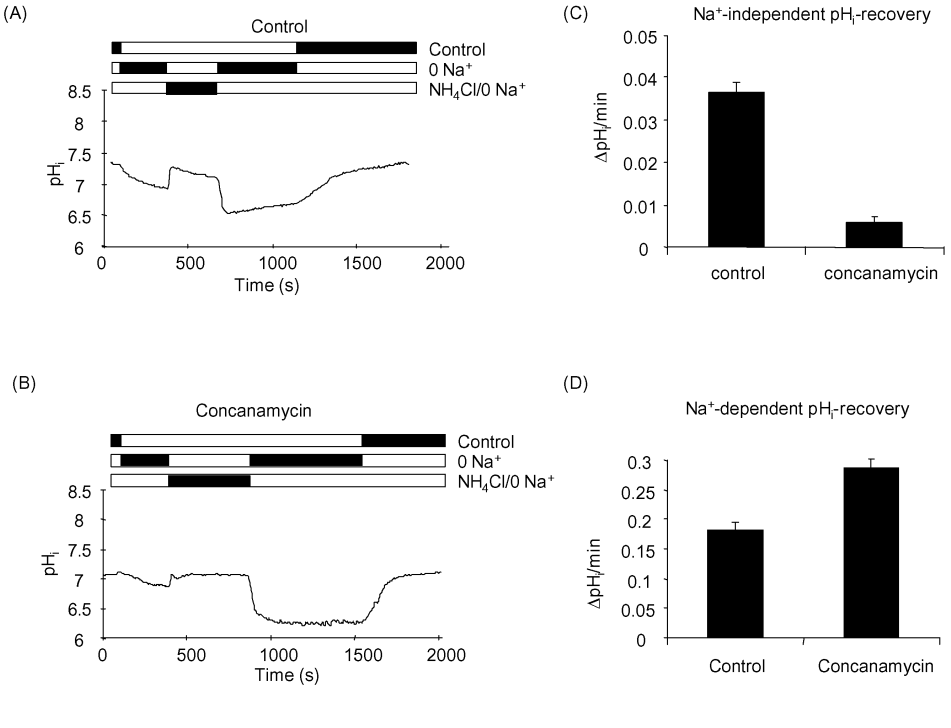


**Fig. 5A–D** $Na^+$-independent and -dependent $pH_i$ recovery in intercalated cells of the cortical collecting duct (CCD). **A** Original $pH_i$ tracing from an intercalated cell in a CCD obtained by digestion under control conditions. **B** Original $pH_i$ tracing from an intercalated cell from a digested CCD in the presence of concanamycin (100 nM). **C** Inhibition of $Na^+$-independent $pH_i$ recovery in intercalated cells in the CCD by concanamycin. **D** $Na^+$-dependent $pH_i$ recovery in intercalated cells from the CCD under control conditions and in the presence of concanamycin

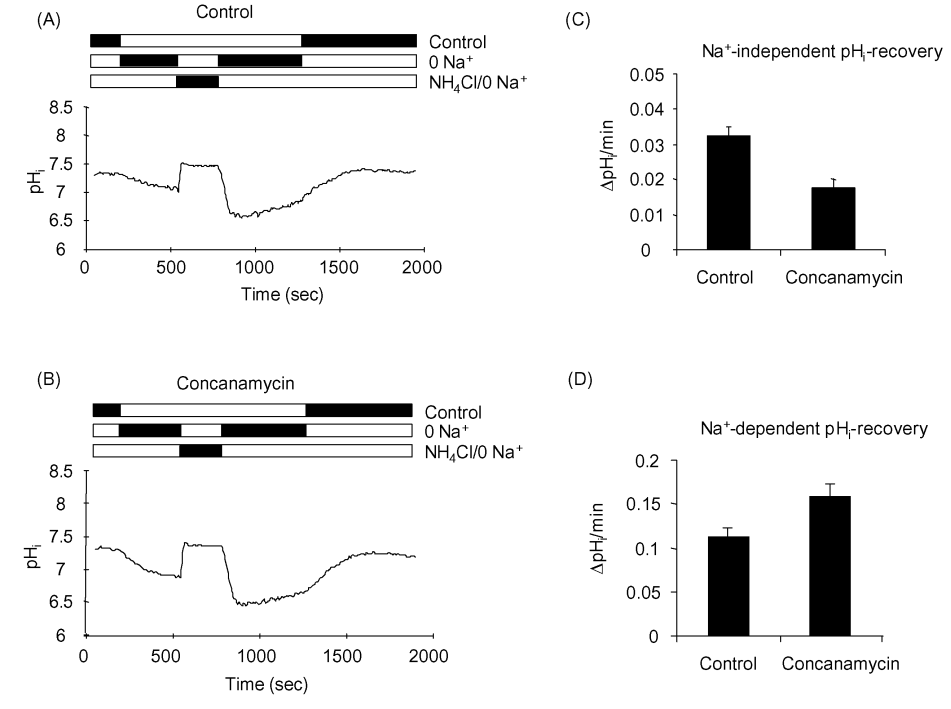


**Fig. 6A–D** $Na^+$-independent and -dependent $pH_i$ recovery in intercalated cells from the outer medullary collecting duct. **A** Original tracing of $pH_i$ from an intercalated cell in an OMCD obtained by digestion under control conditions. **B** Original tracing of $pH_i$ from an intercalated cell in an OMCD obtained by digestion in the presence of concanamycin (100 nM). **C** Inhibition of $Na^+$-independent $pH_i$ recovery in intercalated cells from the OMCD by concanamycin. **D** $Na^+/H^+$ exchanger activity in intercalated cells from the OMCD under control conditions and in the presence of concanamycin

In the present study, we modified the enzymatic method developed by Schafer et al. [38] to isolate rapidly large numbers of tubules from the mouse kidney. These large numbers of tubules will be of considerable interest when employed for the study of genetically altered mouse models. Tubules isolated using our modified method proved to be suitable for studies of single tubules in vitro and therefore allowed us to extend our studies of $H^+$-ATPase activity and regulation in mouse kidney. Morphological and functional analysis demonstrated the usefulness of this simple and rapid method for obtaining large numbers of viable, defined mouse kidney tubule segments. Whereas electron microscopy showed that a considerable portion of unselected proximal tubules were damaged by this procedure, careful examination by light microscopy allowed the selection of intact proximal tubules and collecting ducts.

Immunofluorescence staining for various proteins involved in acid-base transport in the collecting duct

demonstrated that the digestion procedure did not alter significantly membrane expression or polarity. Cold treatment of kidney slices reportedly leads to redistribution of some key membrane transport proteins, including the vacuolar $H^+$-ATPase and aquaporin water channels [6]. However, in the present preparation, an apparently normal distribution of $H^+$-ATPase, AE-1 and pendrin was observed in selected, isolated tubule fragments. This is probably due to the fact that the tubules were allowed to recover at 37 °C for at least 30 min prior to the acute experiment. In addition, the majority of intercalated cells in cortical collecting ducts showed a diffuse or bipolar $H^+$-ATPase localization indicating the presence of numerous B-type intercalated cells in mouse kidney as confirmed by pendrin staining [10, 11, 24, 46].

Functional studies using the pH sensitive dye BCECF showed that selected proximal tubules and collecting ducts were viable for periods up to 2 or 5 h, respectively, when placed in a perfusion chamber in vitro. This time frame allows the use of several tubules from different nephron segments from the same kidney/animal. Loading with the BCECF-AM ester and subsequent concentration of de-esterified dye within the cell depend critically on the integrity of intracellular enzymes and of the cell membrane. In a damaged tubule, after cleavage of the ester bonds, the hydrophilic BCECF would diffuse rapidly out of the cell. Moreover, in proximal tubules, the values that we obtained while measuring intracellular pH, as well as $Na^+/H^+$-exchanger and $H^+$-ATPase activity were comparable to values reported earlier from hand-dissected tubules obtained mainly from the rabbit [17, 26, 55].

$pH_i$ measurements in single intercalated cells in the cortical collecting duct and in collecting ducts from the inner stripe of the outer medulla yielded values for $Na^+/H^+$-exchanger and $H^+$-ATPase activity lower than those previously described for other species [43]. The lower activity may be due to either species differences or the delayed fluid exchange in the collapsed tubule lumen. Future studies using isolated perfused tubules will be needed to examine this question in detail. Inhibition of $H^+$-ATPase activity by the specific V-type $H^+$-ATPase inhibitor concanamycin [16] revealed that more than 80% of $Na^+$- and $HCO_3^-$-independent $pH_i$ recovery in the cortical collecting duct, but only about 50% in the outer medullary collecting duct, was due to $H^+$-ATPase activity. This finding suggests that other $H^+$-extruding mechanisms are present in the latter segment. A potential candidate is an $H^+/K^+$-ATPase, which has been described previously in this segment [53]. In addition, an increase in $Na^+/H^+$-exchanger activity was observed following inhibition of the $H^+$-ATPase in intercalated cells from cortical and outer medullary collecting ducts. This may be due to activation of $Na^+/H^+$-exchange following impairment of the cells to recover their intracellular pH following the acid load in the presence of $H^+$-ATPase inhibitors. Further studies will be needed to address this issue.

Because the experiments described in the present study were performed using collapsed non-perfused tubules, we are presently not able to distinguish between apical or basolateral $H^+$ extrusion. The fact that the exchange of intraluminal fluid is limited under these conditions may also lead to underestimation of apical $H^+$ extrusion through apical $Na^+/H^+$-exchanger isoforms and vacuolar $H^+$-ATPase in the proximal tubule and type-A intercalated cells. Perfusion of isolated tubule fragments will certainly help to clarify these points in future studies.

In summary, we have modified an enzymatic method for digesting mouse kidney to obtain defined nephron segments. Morphological and functional characterization demonstrated the usefulness of this method and the viability of selected tubules for functional experiments. Moreover, we present the first data on the activity of the vacuolar $H^+$-ATPase in isolated mouse tubule fragments.

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